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Characterization of novel marine sulfate-reducing bacteria resistant to RDX and other explosives

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Abstract

Exposure to nitro-compounds like RDX, TNT contained in unexploded ordnance pose significant risks for people and the environment due to their toxicity and explosive nature. Bombing sites used for military training activities contain considerable amounts of these contaminants. Until 2003 and for more than 60 years, the US Navy used the eastern part of Vieques, Puerto Rico as a bombing range. Since then, leaching of explosive compounds like RDX from unexploded ordnance represents a serious threat to the marine ecosystem of the area. The contribution of sulfate-reducing bacteria (SRB) to natural attenuation of explosives has been demonstrated in freshwater environments; however, little is known about their contribution in marine environments. From the contaminated sediments, we isolated 5 novel SRBs using lactate as the sole electron donor. Preliminary 16S rDNA analyses identify all 5 isolates to be delta proteobacteria most closely related to *Desulfovibrio*, with 16S rDNA sequences less than 94% of similarity with known sequences, thus representing putative new species. In general, the isolates grew best at 37°C, at a pH of 7.5 and optimal growth was obtained with 1.5-3% of salt added in the media. While all isolates grew with pyruvate as the alternative electron donor, 3 of the isolates grew with 20mM glycerol, one of them showed growth on 50mM fumarate. Electron acceptors sulfate, sulfite and thiosulfate were reduced by all isolates. Preliminary results indicate two of the isolates could also reduce Mn(IV). Some of the bacterial isolates grew better in explosive-amended media. The growth and survival in the presence of TNT increased with added salinity of the media. Based on this result and the fact that the isolates have had prior exposure to explosives, investigations are underway to study the potential of RDX degradation by the novel isolates.

Site Description and Isolation of strains

Geographic map of the island of Vieques in reference to Puerto Rico and the islands of the Caribbean.

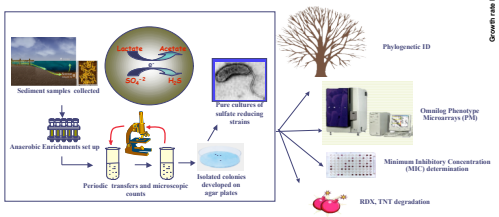
The US Navy gunnery range at Vieques, Puerto Rico was used for nearly 60 years for all types of live weapon fleet training. In 2005 these military facilities were included by the US EPA in the NPL of Superfund sites.

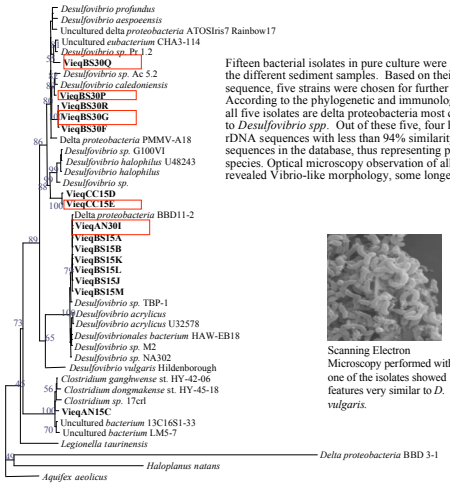
This site inherently possesses a promising opportunity for studying the ecology and physiology of bacteria that have had long-term exposure to explosives in a tropical marine environment. Since microbes at this location have been exposed to nitro compounds for a long time, Sulfate Reducing Bacteria (SRB) with an improved capacity to use these compounds as a source of nitrogen or alternative electron acceptors may have been selected in nature, thus contributing to ongoing attenuation in marine sediments.

Site	Description	Nitrate	Sulphate	Fe(II)	Chloride
Vqa 2	Marine sediment sample from 10m, an inside barrel next to the USS Killens	0.7mM	6.25mM	0mM	95mM
Vq 1	Same as above	0.72mM	0.58mM	0.1mM	28.5mM
Ccn 4	Marine sediment sample from ~3 m, collected next to an UXO	0.29mM	5.1mM	0.10mM	75mM
Af 3	UXO surface sample from ~3 m	0.67mM	5.5mM	0.25mM	77mM
An 5	Black sediment sample from the lagoon complex at the AFWTA	0.8mM	5.1mM	0mM	80.5mM
Ftl	Reference sample from firing point 3	8.4mM	0.58mM	0.20mM	0.5mM

Sediment samples from different sites of the gunnery range was analyzed for their anionic content. All site samples had negligible nitrate content and almost all contained high concentration of chloride and sulfate true to the marine nature of the sediments.

Schematic of the Project Objectives





Phylogeny analysis of strains based on partial 16S rDNA sequence analysis (bootstraps values >45 are shown).

Fifteen bacterial isolates in pure culture were isolated from the different sediment samples. Based on their 16S rDNA sequence, five strains were chosen for further analyses. According to the phylogenetic and immunologic analyses, all five isolates are delta proteobacteria most closely related to *Desulfovibrio spp*. Out of these five, four have 16S rDNA sequences with less than 94% similarity with known sequences in the database, thus representing putative new species. Optical microscopy observation of all of the isolates revealed Vibrio-like morphology, some longer than others.

Scanning Electron Microscopy performed with one of the isolates showed features very similar to *D. vulgaris*.

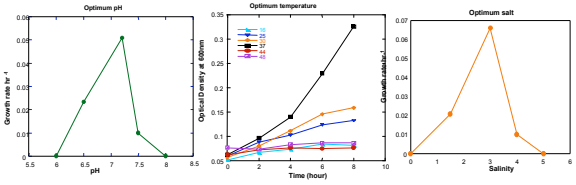
Characterization of the novel isolates

Electron Donor	Strain Vieq Q	Strain Vieq E	Strain Vieq I	Strain Vieq P
Lactate	+	+	+	+
Pyruvate	+	+	+	-
Fumarate	+	-	-	+
Acetate	-	-	-	-
Benzoate	+	-	+	-
Citrate	-	-	-	-
Glucose	-	-	-	-
Formate	-	-	-	-
Glycerol	+	+	-	+
Propionate	-	-	-	-
Succinate	-	-	-	-

Of the different novel isolates, 4 were chosen for further characterization. The isolates exhibit growth on a narrow range of alternative electron donors when sulfate was used as the electron acceptor. In most cases growth was determined as increase in optical density and/or direct cell counts. Interestingly, two of the isolates demonstrated a cell doubling growing on 1mM benzoate, which is the central intermediate of anaerobic aromatic hydrocarbon degradation.

Electron Acceptor	Strain Vieq Q	Strain Vieq E	Strain Vieq I	Strain Vieq P
Sulfate	+	+	+	+
Sulfite	+	+	+	+
Thiosulfate	+	+	+	+
Nitrate	-	-	-	-
Oxygen	-	-	-	+
Cr (VI)	+	+	+	+
Fe (III)	+	+	+	+

With lactate as the electron donor, all isolates utilized sulfate, sulfite, thiosulfate as alternative electron acceptors. This is very typical of sulfate reducing bacteria. However, interestingly, one of the isolates, strain Vieq P showed moderate growth in the presence of oxygen. The isolates also used metals like Iron(III) and Chromium (VI) as electron acceptors.

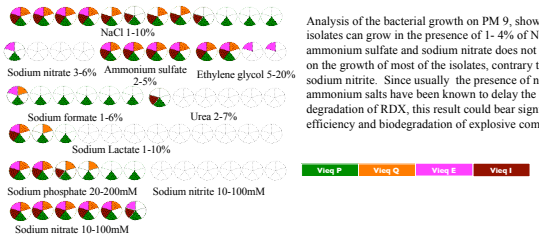


Optimal growth conditions for the strains were also determined as shown here for strain Vieq Q.

Strain	Optimum pH	Optimum salinity	Optimum temperature
Vieq E	8	3%	37°C
Vieq I	7.5	1.5%	37°C
Vieq P	7.2	1.5%	37°C
Vieq Q	7.2	3%	37°C

While all of the isolates grew optimally at 37°C, their optimal salinity and pH requirements differed.

OMNILOG Phenotypic Microarray



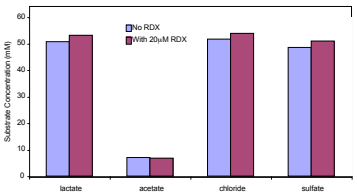
Analysis of the bacterial growth on PM 9, shows that the bacterial isolates can grow in the presence of 1- 4% of NaCl. The presence of ammonium sulfate and sodium nitrate does not seem to have a toxic effect on the growth of most of the isolates, contrary to the results seen with sodium nitrite. Since usually the presence of nitrate, nitrite and ammonium salts have been known to delay the onset of anaerobic degradation of RDX, this result could bear significance to the growth efficiency and biodegradation of explosive compounds by these cultures.

TNT, RDX Studies

Strains	TNT (uM)			RDX (uM)		
	0 % NaCl	1.5 % NaCl	3.0 % NaCl	0 % NaCl	1.5 % NaCl	3.0 % NaCl
VieqP	No growth	60	52	No inhibition	No inhibition	No growth
VieqQ	No growth	88	54	No inhibition	96	No growth
VieqG	No growth	62	63	No inhibition	No inhibition	No growth
Vieq1	35	N/A	45	No inhibition	50	No growth
VieqE	No growth	58	56	No inhibition	No inhibition	No growth
D.vulgaris	49	63	62	96	63	No growth

MIC values were determined for five isolates and the model organism *D vulgaris* with TNT and RDX. It appears that cells of *D. vulgaris* tolerate high levels of TNT at different NaCl concentrations, and in most cases, better than the strains isolated from RDX exposed sites.

The results show that in general, the isolates withstand the presence of RDX in media amended with upto 1.5% NaCl. No growth is observed with any strains at 3% salinity. In contrast, they grow with TNT amendment in media containing upto 3% salinity. At 0 % (w/v) NaCl, they do not grow with TNT but grow optimally with RDX. Interestingly, in media without any added NaCl, while all the isolates exposed to RDX grow optimally, no growth is exhibited when exposed to TNT. There appears to be a correlation between the presence of salt and the effect the specific nitrocompound has on bacterial growth, however, it needs further detailed analysis.



Preliminary experiments were set up to study the effect of RDX on the bacterial growth in media that contains no added nitrogen. Initial results with strain Vieq Q shows that RDX is probably not being degraded enough to serve as the sole nitrogen source. No significant cell number increase is seen in parallel incubations without RDX amendments and subsequently, there is no difference in substrate utilization between the two treatments over a period of 10 days.

Conclusions

- Several sulfate reducing bacterial isolates were obtained from nitro-compound contaminated marine sediment samples at the former US Navy gunnery range at Vieques, Puerto Rico.
- Of these isolates five are less than 94% similar to their closest relatives by 16S rDNA analyses and could represent putative new species.
- Initial characterization shows that all isolates grow optimally at 37°C, and at pH around 7.2-8. Optimal salinity differed between 0-3%.
- Phenotypic microarray analyses revealed that nitrite is toxic to all isolates, while high concentrations of 80 -100mM nitrate is not.
- The isolates were tolerant to significant concentrations of TNT and RDX while growing in salt amended media. The correlation of the MICs of these compounds to the salinity content of the media needs further investigation.
- Preliminary studies are in progress to asses the biodegradation of RDX and TNT by these novel isolates

Acknowledgement

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